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14. ABSTRACT While prostate cancer is an important cause of cancer mortality, most men diagnosed with early prostate cancer experience an indolent course. We are evaluating molecular and clinical predictors at diagnosis to distinguish lethal and indolent prostate cancer. In a related project, we have tested the predictive value of a previously identified multigene tumor signature, a 12-gene model. Risk classification based on the 12-gene model predicted development of lethal disease 20 years hence. The best discrimination came from combining information from the 12-gene markers and clinical data, which perfectly classified the lowest risk stratum where no one died of cancer and provided greater discriminatory ability (AUC 0.78) than the clinical model alone (AUC 0.71), $p=0.04$. We now are testing the 12-gene and additional tumor markers within a prostatectomy cohort of 950 men from the Physicians' Health Study and Health Professionals Follow-up Study. We have constructed high-density tumor tissue microarrays on the cohort, and have undertaken immunohistochemistry to characterize protein expression. We have also completed abstraction of clinical data from medical records and pathology reports. Preliminary analyses and model building to test the discriminatory ability of the gene markers and clinical data are now underway, with the ultimate goal to provide prognostication of lethal and indolent prostate cancer.					
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Introduction

Upon diagnosis with localized prostate cancer, patients and clinicians are faced with the decision of whether to treat or to defer treatment. On one hand, prostate cancer is a leading cause of cancer death among men in westernized countries (1), and deaths occur even 20 years after diagnosis (2). On the other, treatment has adverse effects (3) and is often unneeded, as most men do not die from their cancer, and many harbor tumors which are indolent even in the absence of therapy (2, 4, 5). Thus, there is a clear need for tools to distinguish potentially lethal from indolent disease at diagnosis to guide treatment decisions. In the current project, we seek to test molecular and clinical predictors at diagnosis to discriminate lethal and indolent prostate cancer within two large US cohorts of prostate cancer cases from the Physicians' Health Study (PHS) and the Health Professionals' Follow-up Study (HPFS). In Aim 1, we will develop the best set of clinical markers to predict aggressive prostate cancer, as defined by development of distant metastases or prostate cancer death. In Aim 2, we will develop a composite biomarker to predict aggressive prostate cancer, and quantify the extent to which these markers, in combination with clinical parameters, can discriminate aggressive from indolent disease.

Body

Aim I. To determine clinical predictors of aggressive prostate cancer.

We have undertaken an abstraction of clinical data from medical records and pathology reports for the 6,040 prostate cancer cases in the Physicians' Health Study (PHS) and Health Professionals' Follow-up Study (HPFS). We have retrieved information on tumor grade, stage, extent, PSA levels at diagnosis, clinical presentation and treatments. The abstraction of clinical parameters is 95% complete. A copy of the coding sheet appears as **Appendix 1**.

A clinical database in SAS has been constructed including the cases for which abstraction of clinical parameters is complete. Quality control assessment of this database is currently underway, and we have shown high quality of the data. The clinical database from the prostate cancer cases has been merged with additional data files, including death files and questionnaire data, from the PHS and HPFS cohorts. The quality assurance of the merged data has been assessed and variables needed for future analyses have been coded and added to the data files. The final analysis data file will be made available on the UNIX server when information has been abstracted from the remaining 5% of cases.

Preliminary statistical analyses have begun in order to develop a model of clinical parameters that predicts prostate cancer death. Using Cox proportional hazard models, we have found Gleason grade at diagnosis to be one of the strongest predictors of lethal and indolent prostate cancer, with relative risks of 3.8 ($p<0.001$) and 7.1 ($p<0.001$) for Gleason 7 and Gleason 8-10 tumors compared to Gleason 2-6. Among men with Gleason 7 tumors, there was no prognostic difference between Gleason 3+4 versus 4+3 tumors, an observation noted by Andren et al(6). We also observed a significant association between older age at diagnosis ($p<0.001$) and higher psa levels at diagnosis ($p<0.001$) with development of lethal prostate cancer.

For approximately 1,100 cases in the PHS and HPFS cohorts, we have collected information on Gleason grade through a standardized Gleason scoring by the pathologist on our project. We are now comparing the re-review Gleason with the original Gleason from pathology reports, and have found evidence that about 20% of the standardized scores underwent a shift (upgraded or downgraded) towards final Gleason scores. This grade migration has been previously reported by Albertsen et al(4). In further analyses, we will evaluate the prognostic ability of Gleason, comparing the original and re-reviewed Gleason scores.

Aim II. To develop a composite set of molecular predictors of aggressive prostate cancer by a multiplex approach.

Much of the effort on this aim to date has been dedicated to developing the tumor tissue biobank and construct consisting of diagnostic and prostatectomy tumor specimens from the HPFS and PHS prostate cancer cohorts. We have collected archival materials for 1,083 HPFS cases and 1,125 PHS cases, and a tissue tracking system has been developed for the PHS and HPFS tumor repository.

The prostate cancer cases in the PHS and HPFS tumor repository have undergone pathologic review to obtain standardized review of Gleason grade, tumor extent, tumor type, and other histological features. The tumor tissue in each specimen has also been identified and marked in preparation for the construction of tissue microarrays (TMA). To present, tumor tissue microarrays have been constructed from prostatectomy specimens for a total of 670 HPFS cases on five TMAs and 450 PHS cases on four TMAs. A fifth TMA for the PHS and HPFS is in the process of review and construction.

Among the proposed biomarkers, p63, MTA1, Jagged1, Amacr, and ABP280 have already been stained by immunohistochemistry and evaluated for intensity and protein expression using the Chromavision Imaging system. Staining for KI67 is almost complete, and the remaining biomarkers are planned for the near future. In preliminary analyses, we have evaluated the relation between three individual biomarkers (**Table 1**) and prostate cancer death within the PHS cohort using proportional hazard models. The data in Table 1 are adjusted for the clinical factors age at diagnosis and Gleason score. Although the number of events are still small, the data suggest that tumor tissue expression of key biomarkers may be predictive of development of lethal prostate cancer, and could be useful together as a signature in prognostication of indolent and lethal disease.

Biomarker	Hazard ratio (95% CI), comparing highest versus lowest expression	P value
MTA1	5.5 (0.8-37.4)	0.08
P63	1.7 (1.0-2.7)	0.05
AMACR	2.1 (1.1-3.0)	0.03

These data are in line with a complementary study from our group assessing the 12-gene model to predict lethal prostate cancer within the Swedish Watchful Waiting Cohort (**Appendix 2**). This study provides additional support for the current project.

Key Research Accomplishments

- Developed Clinical database for 6,040 prostate cancer cases in the Physicians' Health Study (PHS) and Health Professionals' Follow-up Study
- Assembled tumor tissue repository of biopsy, TURP and prostatectomy specimens for 2,208, including tracking system
- Finished standardized histopathologic review of tumor tissue cohort and constructed 9 tumor tissue microarrays for the PHS and HPFS cohorts
- Completed immunohistochemical staining and evaluation for five biomarkers on the TMAs, and assembled biomarker database
- Initiated statistical analyses to identify clinical and molecular predictors to distinguish indolent and lethal prostate cancer
- Summarizing results for submission as Abstracts to cancer and pathology research conferences

Reportable Outcomes

- Submission of an abstract on AMACR expression and prostate cancer death for the US and Canadian Academy of Pathology Meeting, March 2008
- Preparation of an abstract on prognostication of Gleason grade shifts and survival for the American Society of Clinical Oncology Genitourinary Cancer Symposium, February 2008
- Preparation of an abstract on p63 tumor expression and clinical correlates for the American Association of Cancer Research Frontiers in Cancer Prevention Meeting, December 2007
- Presented poster at the 2007 DoD ImPACT Meeting in Atlanta
- Invited as faculty speaker at the Prouts Neck Prostate Cancer Meeting to be held November 2008
- Development of prostate tumor tissue repository of biopsy, TURP and prostatectomy specimens
- Received Development Project Award from Dana Farber/Harvard Cancer Center Prostate Cancer SPORE program related to this work
- Data generated from this project will provide thesis data for one doctoral student at Harvard School of Public Health
- PI was promoted to Assistant Professor of Medicine at Harvard Medical School and Assistant Professor of Epidemiology at Harvard School of Public Health based on experience supported by this award

Conclusion

Our preliminary results provide a proof of concept regarding the use of clinical and tumor biomarkers at diagnosis to predict prognosis several years in the future. The data suggest that evaluation of these data can enhance prediction models to aid in counseling patients and guide clinical practice. The future challenge is to improve molecular signatures so that a greater proportion of men can be classified as low or high risk with similar or better discrimination. In addition to the proposed biomarkers, future work may seek to improve the signature with addition of data on germline variation in key genes, somatic alterations in genes, or circulating biomarkers. The PHS and HPFS cohorts are rich in these biomarker data, and could be added to the project in a time efficient manner. Another future goal would be to identify additional prostate cancer cohorts on which to replicate our study findings.

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Appendices

1. Coding Sheet for abstraction of clinical data from medical records
2. Manuscript on 12-gene model and lethal prostate cancer assessed within the Swedish Watchful Waiting Cohort

1. **PRESENTATION:** ☐ symptoms ☐ PSA screening ☐ other
☐ DRE, abnormal ☐ metastatic symptoms ☐ unspecified
2. **DIAGNOSTIC:** ☐ needle biopsy ☐ other surgical approach ☐ unspecified
☐ TURP ☐ aspiration cytology
3. **BIOPSY** Date: / ☐ Unknown date ☐ Did not have biopsy
month year
- b. # Cores biopsied: # Cores with cancer: ☐ unspecified

4. STAGING INFORMATION:

CLINICAL

PATHOLOGICAL

DRE at diagnosis	<input type="radio"/> normal <input type="radio"/> abnormal <input type="radio"/> unspecified	
Capsule	<input type="radio"/> intact <input type="radio"/> invasion <input type="radio"/> perforation <input type="radio"/> unspecified	<input type="radio"/> intact <input type="radio"/> invasion <input type="radio"/> perforation <input type="radio"/> unspecified
Seminal vesical involvement	<input type="radio"/> yes <input type="radio"/> no <input type="radio"/> possible <input type="radio"/> unspecified	<input type="radio"/> yes <input type="radio"/> no <input type="radio"/> possible <input type="radio"/> unspecified
Metastases	Bone: <input type="radio"/> yes <input type="radio"/> no <input type="radio"/> suspicious <input type="radio"/> unspecified	Lymph Nodes: <input type="radio"/> yes <input type="radio"/> no <input type="radio"/> suspicious <input type="radio"/> unspecified
Jewett-Whitmore Stage	<input type="radio"/> A <input type="radio"/> B <input type="radio"/> C <input type="radio"/> C1 <input type="radio"/> C2 <input type="radio"/> D <input type="radio"/> D1 <input type="radio"/> D2 <input type="radio"/> unspecified	<input type="radio"/> A <input type="radio"/> B <input type="radio"/> C <input type="radio"/> C1 <input type="radio"/> C2 <input type="radio"/> D <input type="radio"/> D1 <input type="radio"/> D2 <input type="radio"/> unspecified
TNM Stage	<input type="radio"/> T1 <input type="radio"/> T1a <input type="radio"/> T1b <input type="radio"/> T1c <input type="radio"/> T2 <input type="radio"/> T2a <input type="radio"/> T2b <input type="radio"/> T2c <input type="radio"/> T3 <input type="radio"/> T3a <input type="radio"/> T3b <input type="radio"/> T3c <input type="radio"/> T4 <input type="radio"/> unspecified	<input type="radio"/> T1 <input type="radio"/> T1a <input type="radio"/> T1b <input type="radio"/> T1c <input type="radio"/> T2 <input type="radio"/> T2a <input type="radio"/> T2b <input type="radio"/> T2c <input type="radio"/> T3 <input type="radio"/> T3a <input type="radio"/> T3b <input type="radio"/> T3c <input type="radio"/> T4 <input type="radio"/> unspecified
	<input type="radio"/> N0 <input type="radio"/> N1 <input type="radio"/> NX <input type="radio"/> unspecified	<input type="radio"/> N0 <input type="radio"/> N1 <input type="radio"/> NX <input type="radio"/> unspecified
	<input type="radio"/> M0 <input type="radio"/> M1 <input type="radio"/> MX <input type="radio"/> unspecified	<input type="radio"/> M0 <input type="radio"/> M1 <input type="radio"/> MX <input type="radio"/> unspecified
	<input type="radio"/> unspecified	<input type="radio"/> unspecified

5. **GRADE AT DIAGNOSIS:** ☐ well-differentiated ☐ moderate ☐ poorly differentiated ☐ unspecified6. **GLEASON AT DIAGNOSIS:** Major: Minor: Total: Gleason unspecified: ☐ unspecified: ☐ ☐ ☐

7. PSA AT DIAGNOSIS: . ng/ml ☐ elevated ☐ normal ☐ unspecified

8. DID PATIENT HAVE A PROSTATECTOMY? ☐ yes ☐ no ☐ unknown

a. DATE OF PROSTATECTOMY: / ☐ unknown
month year

b. GLEASON AT PROSTATECTOMY: Major: Minor: Total:
Gleason unspecified: ☐ unspecified: ☐ ☐ ☐

9. TREATMENT #1: ☐ radiation ☐ prostatectomy ☐ chemo or hormone therapy
☐ orchiectomy ☐ watchful waiting
☐ other Specify:
☐ unspecified

10. TREATMENT #2: ☐ radiation ☐ prostatectomy ☐ chemo or hormone therapy
☐ orchiectomy ☐ watchful waiting
☐ other Specify:
☐ unspecified

CODER 1: ☐ LAM ☐ JL ☐ HL
☐ Other

DATE CODED:

/ /
month day year

CODER 2: ☐ LAM ☐ JL ☐ HL
☐ Other

DATE CODED:

/ /
month day year

Testing a Multigene Model to Predict Lethal Prostate Cancer

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ABSTRACT:

Purpose: While prostate cancer is a leading cause of cancer death, most men die with and not from their disease, underscoring the urgency to distinguish potentially lethal from indolent prostate cancer. We tested the prognostic value of a previously identified multigene signature of prostate cancer progression to predict cancer-specific death.

Methods: The Örebro Watchful Waiting Cohort included 172 men with localized prostate cancer of whom 40 developed lethal disease. We quantified protein expression of the markers in tumor tissue by immunohistochemistry, and stratified the cohort by quintiles according to risk classification. We accounted for clinical parameters (age, Gleason, nuclear grade, tumor volume) using Cox regression, and calculated Receiver Operator Curves to compare discriminatory ability.

Results: The hazard ratio of prostate-specific death increased with increasing risk classification by the multigene model, with a 16-fold greater risk comparing highest versus lowest risk strata, and predicted outcome independent of clinical factors ($p=0.002$). The best discrimination came from combining information from the multigene markers and clinical data, which perfectly classified the lowest risk stratum where no one died of cancer; using the two lowest risk groups as referent, the hazard ratio (95% confidence interval) was 11.3 (4.0-32.8) for the highest risk group and the cumulative incidence difference at 15 years was 60% (50-70%). The combined model provided greater discriminatory ability (AUC 0.78) than the clinical model alone (AUC 0.71), $p=0.04$.

Conclusions: Molecular tumor markers can add to clinical parameters to help distinguish lethal and indolent prostate cancer, and hold promise to guide treatment decisions.

INTRODUCTION

Upon diagnosis with localized prostate cancer, patients and clinicians are faced with the decision of whether to treat or to defer treatment. On one hand, prostate cancer is a leading cause of cancer death among men in westernized countries ¹, and deaths occur even 20 years after diagnosis ². On the other, treatment has adverse effects ³ and is often unneeded, as most men do not die from their cancer, and many harbor tumors which are indolent even in the absence of therapy ^{2,4,5}.

Treatment of localized disease can reduce cancer-specific mortality, but in the only randomized trial of radical prostatectomy versus watchful waiting ^{6,7}, the number needed to treat to prevent one cancer death was 19. That trial predated screening by prostate specific antigen (PSA); 30-60% of PSA-detected cancers have been characterized as over diagnosed ^{8,9} and therefore the number needed to treat may be greater for a screened population.

There is a clear need for tools to distinguish potentially lethal from indolent disease at diagnosis to guide treatment decisions. Clinical nomograms characterize risk of progression using pretreatment clinical markers: PSA levels, biopsy Gleason scores, tumor extent, and clinical stage ^{10,11,12,13}. These scoring systems have significant predictive power, but molecular tumor markers hold promise to improve prediction ¹⁴. A 12-gene molecular signature of advanced prostate cancer was recently identified through integration of proteomic and expression array data, comparing benign prostate, localized prostate cancer and metastatic disease. ¹⁵ A set of 36 markers, which showed differential expression at both the RNA and protein level, plus five additional genes, were immunostained on a prostate cancer progression array. Through linear discriminant analysis, the multigene model was identified which was significantly associated with PSA-failure after prostatectomy in a small cohort. However, most men with PSA recurrence do not develop metastatic or lethal disease ¹⁶. We tested the

prognostic value of this molecular signature in relation to prostate cancer death within a cohort of men diagnosed with localized prostate cancer and followed prospectively over 28 years.

METHODS

Study population. The population-based Örebro Watchful Waiting Cohort^{2,17} comprises men with localized (T1a/T1b) prostate cancer diagnosed by transurethral resection of the prostate (TURP) for symptomatic benign prostatic hyperplasia. Cases were diagnosed between 1977 and 1991 in Örebro, Sweden, within a catchment area for the University Hospital. In accordance with standard treatment, the men were initially followed expectantly with careful monitoring by clinical exams, laboratory tests and bone scans every 6 months during the first 2 years post-diagnosis, and yearly thereafter. Hormonal therapy was initiated upon demonstrated progression to symptomatic disease. Tumor tissue was available for 172 men included in the current analysis.

Follow-up of the cohort is 100% complete through March 2006. Metastases were diagnosed by bone scan. Deaths were identified using the Swedish Death Register, and medical records were reviewed by the study investigators to confirm cause.

Tissue microarrays. We retrieved archival formalin-fixed, paraffin embedded TURP specimens to construct tissue microarrays (TMA) using a manual tissue arrayer¹⁷. The study pathologists reviewed H&E slides for each case to provide uniform Gleason grading. We found a 30% discordance comparing Gleason scoring re-review with the initial pathology review, with generally lower scores in the initial reports. This grade migration has also been described by Albertsen⁴. The pathologist determined the dominant prostate cancer nodule or nodule with the highest Gleason pattern, and two 0.6 mm tissue cores from tumor areas were transferred to the recipient array blocks.

Tumor biomarkers. We assayed protein expression of the markers (Table 1) in the multigene model on the Örebro Watchful Waiting TMA using immunohistochemistry. The TPD52 antibody could not be obtained; thus 11 markers were assessed. One 5 micron section of the TMA block was cut for each protein. Incubations and dilutions for

each antibody were optimized while minimizing background (Table 1). Secondary antibodies linked to streptavidin-biotin were used to visualize staining.

Protein expression was determined on scanned digital images of TMA cores¹⁸ using a semi-automated image analysis system (Chromavision) with high reproducibility¹⁹ that assessed staining intensity (0-255) and percent of positive stained area (0-100%). The study pathologist electronically circled areas of histologically recognizable prostate cancer to capture tumor expression.

Presence of the TMPRSS2:ERG fusion was previously evaluated on a subset of cases (N=107) using a fluorescence in situ hybridization (FISH) assay, as well as by 5' RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE) analysis and sequencing of the reverse-transcription–polymerase chain reaction (RT–PCR) product.²⁰ In an earlier publication of the cohort, we showed that presence of the TMPRSS2:ERG fusion was associated with an almost 3-fold increased risk of cancer death.²⁰

TMA blocks were constructed without prior knowledge of clinical outcomes, and the pathologist remained blinded to outcome during immunohistochemistry evaluation.

Statistical analysis. *A priori* we used protein intensity for markers that stained primarily in the cytoplasm and percent staining for those staining primarily in the nucleus (Table 1). For individuals missing data on specific markers, we imputed using the k Nearest Neighbor classification with k equal to 3 and assigned the mean value among the nearest 3.

To create the molecular signature score, we divided expression of each marker into quartiles, based on the cohort distribution. For markers whose expression is upregulated in metastatic vs. localized cancer, based on prior data (Table 1), we assigned a score = 1 for those in the highest quartile of expression, and 0 otherwise. For markers with downregulated expression, a score = 1 was given for those in the

lowest quartile of expression and 0 otherwise. We calculated a weighted risk score across the 11 markers, multiplying the protein expression coding values (0 or 1) for each gene by the coefficients from the linear discriminant analysis¹⁵, thereby prioritizing genes that provided the greatest discrimination in the original article.

We also created a weighted risk score that incorporated clinical predictors: age at diagnosis (continuous), Gleason grade (categorically, 2-5, 6, 7, 8-10), nuclear grade (categorically, grade I, II, III) and tumor extent (defined categorically as the proportion of chips with tumor, <5%, 5-24.9%, 25-49.9%, 50%+).¹⁷ Finally, we created a combined risk score of the multigene molecular signature and clinical markers to examine their joint predictive value. Men were then classified as having high, intermediate or low risk of lethal cancer based on their molecular signature score, their clinical risk score or combined clinical/molecular risk score divided into quintiles. In separate analyses, we added the TMPRSS2:ERG fusion status to the molecular and molecular-clinical risk scores to evaluate the additional informativeness of the fusion, in combination with other markers, to predict poor cancer prognosis among the subset of men.

We used time-to-event analyses to evaluate the gene signature to predict lethal prostate cancer. Person-time was calculated from date of cancer diagnosis to development of metastases, cancer death or censored at time of death from other causes or end of follow-up (March 2006). Hazard ratios (HR) and cumulative incidence differences (with 95% confidence intervals (CI)) were used as effect measures using the Cox proportional hazards model.

Competing causes of death could play an important role in prostate survival analyses. Men who died of another cause soon after diagnosis are less informative about prognosis, since some would have progressed had they lived longer. We categorized men as: lethal phenotype (men who developed metastases during follow-up, n= 40), indolent phenotype (men who lived at least 10 years after diagnosis without

metastases, n=49), and indeterminate phenotype (men who died of competing causes within 10 years of diagnosis, n=83), and compared clinical characteristics of the three groups. We estimated the cumulative incidence of lethal disease accounting for competing risks ²¹ using a publicly available SAS macro ²². Rather than treating other causes of death as censored observations, this method simultaneously analyzes multiple cause-specific hazards. We fit Cox models stratified by failure type (lethal cancer vs. other cause of death) and adjusted for clinical covariates.

In addition to estimating hazard ratios and cumulative incidence differences, we calculated Receiver Operator Curves (ROC) for the three models – the molecular signature, clinical model, combined molecular-clinical model-- plotting sensitivity versus 1 – specificity to predict lethal disease at 15 years. We compared the Area Under the Curve (AUC), where a value of 1.0 indicates perfect discrimination and 0.5 is no better than chance alone ²³.

Analyses were undertaken using the SAS Statistical Analysis (Version 9.1). The research protocol was approved by the institutional review boards at the collaborating US and Swedish institutions.

RESULTS

Of 172 men with localized prostate cancer, 40% had high grade tumors and 19% had tumor volume greater than 25% (Table 2). During 28 years of follow-up, 40 men died of cancer (N=39) or were alive with metastases (N=1), while 49 were long-term survivors who lived >10 years after their diagnosis without development of metastases. Mean follow-up to development of metastatic disease was 7.6 years (range 0.1-27.1), and from metastasis to death was 2.0 years.

Men who died of prostate cancer tended to have tumors with higher Gleason grade, higher nuclear grade, and greater tumor extent than men with the indolent phenotype (Table 2). Men with lethal phenotype were also more likely to have fusion positive tumors. Men classified as indeterminate had clinical characteristics between lethal and indolent phenotypes, reflecting in this group a mixture of men with indolent and those who would have developed lethal disease if they lived long enough.

Expression of Jagged1 and MTA1 were most strongly correlated with expression of other markers, showing correlation coefficients of 0.3 to 0.4 for positive correlations and -0.3 to -0.4 for inverse correlations; no one marker was correlated with all others. We evaluated each specific marker to predict lethal prostate cancer, adjusted for clinical parameters. The strongest molecular predictors (HR, 95% CI) of lethal prostate cancer were MTA1 (3.4, 1.2-9.2), p63 (1.8, 0.8-4.2), jagged1 (1.8, 0.7-4.5) and ABP280 (1.6, 0.7-3.6). Interestingly, these markers were among the strongest discriminators of metastatic vs. localized disease in the publication by Bismar et al¹⁵.

Using the molecular markers, the age-adjusted hazard ratio of lethal disease increased with increasing risk group classification, with a 16-fold increased risk of cancer death comparing the highest versus lowest risk groups (Table 3). The multigene signature remained a significant predictor of prostate cancer death even controlling for clinical parameters: the hazard ratio of developing lethal disease was 12.3 (95% CI 1.5-

100.7) comparing extreme risk groups, and there was increased risk for all risk categories compared to the lowest (p for trend = 0.0015). Moreover, the molecular signature was a significant predictor of lethal disease among men with low grade (Gleason score 4-6) tumors (HR = 16.9, $p=0.007$).

Gleason grade, tumor volume and nuclear grade were each independent predictors of prostate cancer prognosis. Men classified at highest risk based on the clinical markers were 13 times (95% CI 4.3-40.5) more likely to die of prostate cancer compared to the lowest risk group (Table 3). Interestingly, among men characterized as low or intermediate risk based on clinical parameters, the multigene signature could further stratify who would have good or bad prognosis (p for trend 0.028).

While both the molecular and clinical signatures independently predicted cancer death, the best discrimination came from a score combining the multigene and clinical information. No man classified as lowest risk in the combined score died of his disease (Table 3). Given no deaths in the lowest risk group, we combined the two lowest risk strata as the referent category to calculate hazard ratios. With this comparison, the hazard ratio of developing lethal prostate cancer was 11-fold higher (95% CI 4.0-32.8).

Figure 1 shows cumulative incidence of lethal prostate cancer at 5, 10, 15 and 20 years of follow-up based on risk according to the combined multigene and clinical parameters. Even at 5-years, higher risk groups identified those who developed lethal disease (cumulative incidence difference 28.7%, 95% CI 17.4-40.0%). With continued follow-up, the difference in cumulative incidence of lethal cancer between the lowest and highest risk group increased. Although the greatest discrimination in prediction was in contrasting the highest and lowest risk groups, the intermediate risk groups also were predictive of outcome.

ROC curves are presented in Figure 2. At 15 years follow-up, the predictive ability of the molecular signature alone (AUC 0.68) was similar to that of the clinical

markers alone (*AUC* 0.71). The model that combined the molecular and clinical parameters provided the greatest discrimination (*AUC* 0.78), with a 10% improvement over the clinical markers alone ($p=0.04$). The highest risk score based on clinical parameters was a better classifier (higher sensitivity) than the molecular signature of those who would develop lethal disease. However, 10% of the lowest risk men based on clinical markers died of cancer during follow-up, compared to 3% classified as low risk by the molecular signature and 0% classified by the molecular-clinical model, suggesting the molecular data could improve classification of those who would have a good prognosis.

Among the subset of 107 men, information onTMPRSS2:ERG fusion status improved prognostication of the multigene model. At 15-years follow-up, the *AUC* for the multigene signature + fusion data was 0.79, and for the combined molecular/clinical + fusion data was 0.83.

DISCUSSION

In this population-based cohort of men with initially untreated localized prostate cancer, we tested and validated a proposed multigene signature to predict death from disease or long term survival. The overall probability of developing lethal prostate cancer was 1 in 5. The multigene model was a significant predictor of cancer prognosis, independent of clinical parameters, such that the probability of developing lethal disease was 1 in 20 for those classified at lowest risk, but 1 in 2 for those classified as highest risk. The signature distinguished lethal and indolent disease even among men with tumors Gleason <7. These data demonstrate that tumor markers at diagnosis can predict outcome more than 20 years hence, and suggest that in part the biologic phenotype of prostate tumors to have a lethal or indolent course is set early in the disease development.

The discriminatory ability of the molecular signature and clinical model were similar based on the ROC curves. However, the clinical model was a worse classifier for the low-risk group, and misclassified a greater proportion of men as indolent who in reality died of their disease. In assessing classification, one should consider misclassification of truly lethal disease to be a more hazardous occurrence.

The combination of molecular and clinical data provided the greatest outcome discrimination. None of the lowest risk men (20% of the total) developed lethal disease, whereas by the end of follow-up, almost three-quarters of those classified as highest risk had died of their cancer. While few would suggest active surveillance for a man diagnosed with Gleason 8 or higher tumors, molecular markers may be most informative in guiding treatment decisions among men with Gleason 6-7 tumors or where other clinical parameters are suggestive of low to mid risk. The improvement in the AUC for the combined multigene/clinical model compared to the clinical model alone

suggests that prostate cancer prediction models may seek to combine both molecular and clinical data.

These data provide a proof of concept and demonstrate the potential utility of molecular signatures of prostate cancer death. The signature was imperfect, however; not all men with the multigene signature died of the disease. Moreover, the majority of deaths occurred in the middle risk groups, with mixed discriminatory ability, reflecting the need for better markers to classify outcomes. Nonetheless, the ability to predict accurately a man's outcome from prostate cancer at the extreme quintiles could be of great clinical utility. Moreover, our suggested that the recently identified TMPRSS2:ERG fusion may provide even greater improvement in prognostication, in combination with other markers. A set of molecular markers has the added potential benefit of being developed into a standardized and objective test. Clinical parameters such as Gleason grading involve a level of subjectivity, as demonstrated in the apparent Gleason score reclassification which has occurred over time⁴.

For validation of biomarkers of prostate cancer prognosis, cancer-specific death is the optimal outcome. While PSA recurrence is associated with an increased risk of prostate cancer death, most men with recurrences do not die of cancer^{24,25}, so studies based on intermediary measures may be misleading. Long-term and complete follow-up is critical, since prostate-specific deaths can occur even 20 years after diagnosis^{2,4}. The Örebro cohort has been followed prospectively with careful clinical annotation².

The cohort was followed by watchful waiting, and thus initially treatment naïve, which provides an opportunity to characterize a man's cancer as indolent even in the absence of therapy. Our study population derived from a well-defined catchment area, with similar clinical care for all patients, thus reducing potential selection biases. We applied a standardized histopathologic review for Gleason grading to avoid potential grade migration over time⁴. Although the Örebro cohort was assembled in the pre-PSA

era, the cancers were incidentally detected and likely resemble PSA detected cases given the distribution of Gleason grade and stage. These TURP-detected tumors tended to be in the transitional zone, as opposed to peripheral tumors, but there is little evidence to suggest meaningful differences in the biology of tumors in these zones. Indeed, the multigene signature, developed on primarily peripheral zone specimens, was predictive of outcome among our cohort. We had no baseline PSA levels, a clinical predictor of outcome²⁶⁻²⁸, and such information would likely provide a small improvement²⁹ in the predictive probability of the multigene/clinical risk score.

Our findings suggest that evaluation of prostate tumor biomarkers at diagnosis can enhance prediction models to aid in counseling patients and guide clinical practice. The signature can identify men at lowest risk of progression, for whom active surveillance may be most appropriate. Although prediction of the middle risk group is not perfect, the molecular tools can identify men for whom aggressive therapy would be indicated and thus substantially reduce the number needed to treat to avoid one prostate cancer death. The future challenge is to improve the molecular signature so that a greater proportion of men can be classified as low or high risk with similar or better discrimination.

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Figure Legend

Figure 1. Cumulative incidence of lethal prostate cancer during follow-up, based on the combined multigene and clinical risk score. Men were stratified into risk groups based on the combined score, divided into quintiles. Cumulative incidence curves are produced from the proportional hazard models accounting for competing causes of death and age at diagnosis.

Figure 2. Receiver operator curves of the multigene and clinical models to predict development of lethal prostate cancer. The predictive value of the models were assessed by plotting sensitivity versus $1 - \text{specificity}$ to predict prostate cancer death at 15 years, and calculating the area under the curves. The combined molecular signature provided the greatest prognostic discrimination.

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Table 1. Summary of biomarkers in the multigene model¹

Marker	Regulation²	Staining³	CLONE	DILUTION	ANTIGEN RETRIEVAL	SOURCE
AMACR	–	Cytoplasm	13H4 (Rabbit Monoclonal)	1:25	Pressure Cooking Method (PC)	Zeta Corporation
Itga-5	–	Cytoplasm	1 (Mouse Monoclonal)	1:25	Microwave (MW)	BD Biosciences
ABP280	–	Nucleus	5 (Mouse Monoclonal)	1:50	Pressure Cooking Method (PC)	BD Biosciences
CDK7	–	Nucleus	17 (Mouse Monoclonal)	1:50	Microwave (MW)	BD Biosciences
PSA	–	Cytoplasm	(Rabbit Polyclonal)	1:7,500	No Antigen Retrieval Needed	Dako Cytomation
P63	–	Nucleus	4A4 (Mouse Monoclonal)	1:600	Microwave (MW)	Lab Vision Corporation
MTA1	–	Nucleus	A-11 (Mouse Monoclonal)	1:5	Microwave (MW)	Santa Cruz Biotechnology
Kanadaplin	–	Cytoplasm	49 (Mouse Monoclonal)	1:200	Microwave (MW)	BD Biosciences
Jagged1	+	Cytoplasm	(Rabbit Polyclonal)	1:50	Pressure Cooking Method (PC)	Santa Cruz Biotechnology
MIB1	+	Nucleus	MIB-1 (Mouse Monoclonal)	1:200	Pressure Cooking Method (PC)	Dako Cytomation
MUC1	+	Cytoplasm	VU4H5 (Mouse Monoclonal)	1:50	Microwave (MW)	Santa Cruz Biotechnology
TPD52⁴	–					

¹ Bismar et al, Neoplasia 2006

² All genes in the multigene model were similarly dysregulated at the proteomic and transcriptomic level: – = downregulated, + = upregulated

³ Staining stronger in the nucleus than cytoplasm

⁴ Polyclonal antibody for TPD52 not available

Table 2. Characteristics of Örebro Watchful Waiting Cohort, 1977-2005

	Prostate cancer prognosis			
	Overall	Lethal outcome ¹	Indeterminate outcome ²	Indolent cancer ³
N	172	40	83	49
Mean age at diagnosis, yrs	74.1	72.2	76.6	71.3
Mean follow-up, yrs	7.6	5.2	4.4	14.9
Gleason score, %				
4-5	10 (5.8)	3 (7.5)	1 (1.2)	6 (12.2)
6	88 (51.2)	12 (30.0)	44 (53.0)	32 (65.3)
7	50 (29.1)	13 (32.5)	27 (32.5)	10 (20.4)
8-9	24 (14.0)	12 (30.0)	11 (13.3)	1 (2.0)
Tumor Extent, %				
<5	67 (39.0)	8 (20.0)	30 (36.1)	29 (59.2)
5-24.9	72 (41.9)	19 (47.5)	34 (41.0)	19 (38.8)
25-49.9	9 (5.2)	3 (7.5)	5 (6.0)	1 (2.0)
50+	24 (14.0)	10 (25.0)	14 (16.9)	0 (0.0)
Nuclear grade, %				
I	120 (69.8)	21 (52.5)	58 (69.9)	41 (83.7)
II	39 (22.7)	13 (32.5)	19 (22.9)	7 (14.3)
III	13 (7.5)	6 (15.0)	6 (7.2)	1 (2.0)
TMPRSS2:ERG Fusion, %⁴				
Positive	24.4	48.2	14.9	16.1
Negative	75.6	51.8	85.1	84.9

¹ Lethal prostate cancer defined as men who developed distant metastases or died of cancer over follow-up.

² Indeterminant prostate cancer outcome based on short-term follow-up after diagnosis, defined as less than 10 years without development of distant metastases or death from prostate cancer.

³ Indolent cancer based on long-term survival defined as 10 years or more without development of distant metastases or death from prostate cancer.

⁴ TMPRSS2:ERG fusion data was determined for 107 of the 172 men in our study

Table 3. The multigene model as a predictor of lethal prostate cancer: alone and in combination with clinical data, Örebro Watchful Waiting Cohort 1977-2005

	Total N	Lethal N	Hazard ratio (95% CI)
Molecular model only¹			
Quintile 1-Lowest risk	34	1	REF
Q2	35	9	11.8 (1.5-93.8)
Q3-Intermediate risk	34	7	12.3 (1.5-100.7)
Q4	35	13	16.8 (2.2-129.6)
Quintile 5-Highest risk	34	10	16.9 (2.1-133.4)
P for trend			0.0015
Clinical parameters only²			
Quintile 1-Lowest risk	51	4	REF
Q2	11	2	3.3 (0.6-17.9)
Q3-Intermediate risk	51	14	3.7 (1.2-11.3)
Q4	25	4	4.0 (1.0-16.6)
Quintile 5-Highest risk	34	16	13.1 (4.3-40.5)
P for trend			<0.0001
Molecular model and clinical parameters³			
Quintile 1-Lowest risk	34	0	No deaths
Q2	35	5	REF ⁴
Q3-Intermediate risk	34	11	6.3 (2.1-18.3)
Q4	35	11	5.5 (1.9-15.9)
Quintile 5-Highest risk	34	13	11.3 (4.0-32.8)
P for trend			<0.0001

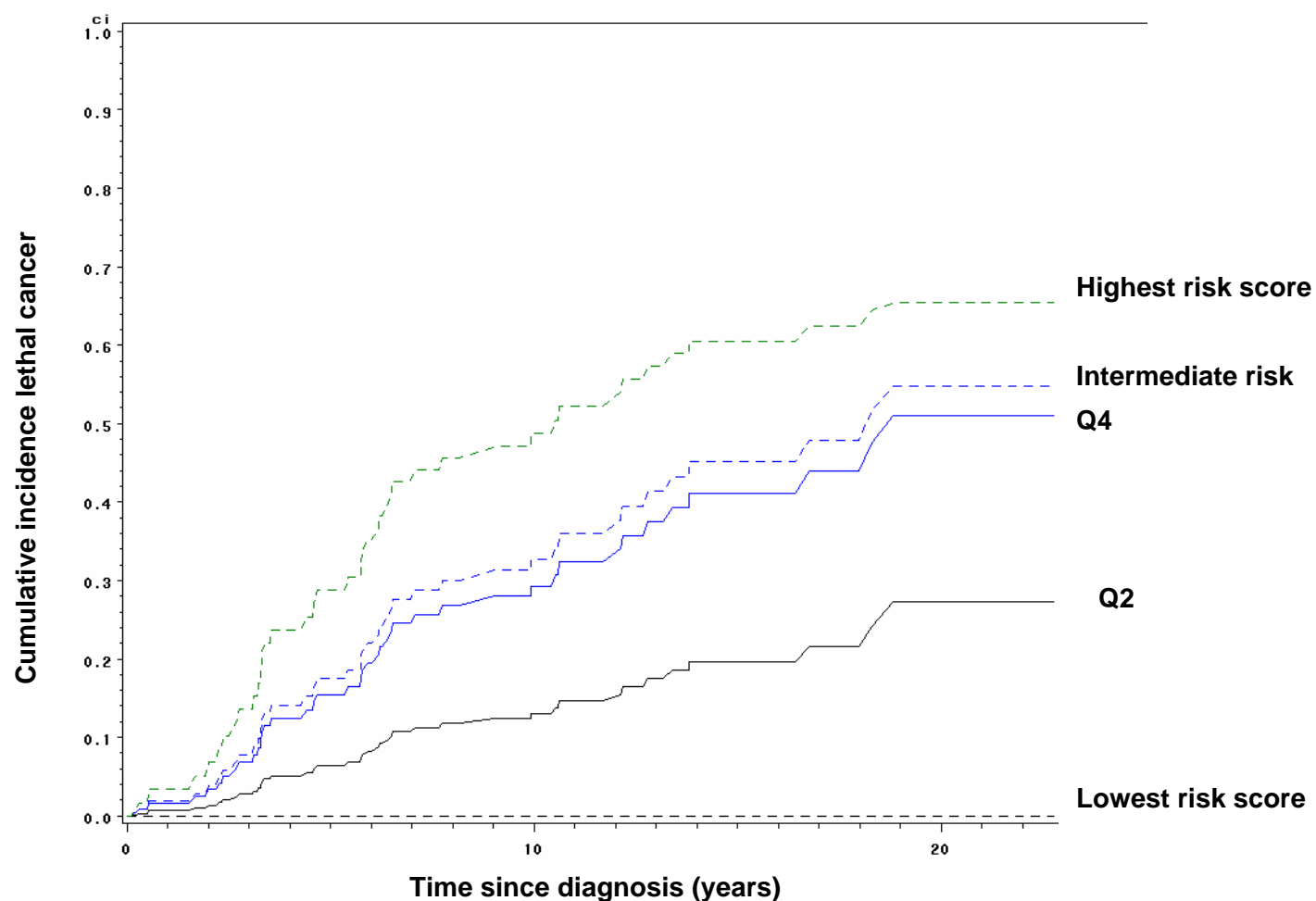
¹ Derived from a linear combination of the indicator variables for protein expression multiplied by the parameters from the linear discriminant model in Bismar et al (2006). Hazard ratios and confidence intervals are adjusted for clinical parameters age at diagnosis, Gleason score, nuclear grade, and tumor extent.

² Derived from a linear combination of indicator variables for the clinical parameters and multiplied by the parameters from the proportional hazards model in Andren et al (2006). Clinical parameters include Gleason grade, nuclear grade, and tumor volume.

³ A linear combination adding together the weighted risk scores for the molecular and clinical models.

⁴ Reference group for this comparison includes men in the two lowest quintiles of risk, since there were no deaths in the lowest risk group.

Figure 1.



Follow-up	Follow-up time			
	5 years	10 years	15 years	20 years
N at risk	106	69	26	4
CI difference				
Lowest risk	REF	REF	REF	REF
Highest risk	+28.7% (17.4-40.0)	+48.7% (37.2-60.2)	+60.4% (50.3-70.5)	+65.4% (57.2-73.6)

Figure 2.

